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Effect of template secondary structure on the inhibition of HIV-1 reverse transcriptase by a pyridinone non-nucleoside inhibitor

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ABSTRACT

The importance of RNA secondary structure on HIV-1 reverse transcriptase catalyzed polymerization and on the potency of the pyridin-2-one inhibitor 3-(4,7-dichlorobenzoxazol-2-ylmethylamino)-5-ethyl-6-methylpyridin-2(1H)-one, L-697,661, were investigated by employing heteromeric primer-template systems. Our data revealed that a stem-loop hairpin secondary structure in the RNA template could lead to strong hindrance of reverse transcription in the reaction catalyzed by HIV-1 reverse transcriptase resulting in the build up of intermediate-length (pause) polymerization products. The presence of L-697,661 greatly enhanced the accumulation of the pause products suggesting that the rate of enzyme translocation from the pause product might be more potently inhibited than polymerization up to the pause site. Model experiments using a synthetic RNA template containing a stem-loop hairpin revealed that the inhibitory potency of L-697,661 increased 2-fold upon polymerization to within four bases of the secondary structure. Inhibitor potency was enhanced over 6-fold when primer-extension proceeded through the duplex region of the stem-loop.

INTRODUCTION

Human Immunodeficiency Virus (HIV) is the etiological agent of Acquired Immune Deficiency Syndrome (1, 2, 3). One of the targets in the development of anti-HIV therapeutics is the enzyme responsible for generation of the proviral DNA from the viral RNA genome. This transcriptional process is essential for viral infectivity and is carried out by the virally-encoded reverse transcriptase (RT; EC 2.7.7.49).

HIV-1 RT catalyzes an array of reactions essential for the successful replication of the virion. The enzyme possesses an RNA- and a DNA-directed DNA polymerase activity (4, 5), an RNase H activity (6, 7, 8), an RNase D activity (9, 10), and a putative strand-transfer activity (11, 12). To date, both nucleoside and non-nucleoside compounds have been prepared as potent inhibitors against viral replication by inhibiting the polymerase activity of RT. The nucleoside inhibitors of HIV-1 RT are DNA chain terminators [e.g., 3′-azidothymidine (AZT) (13)]. As such, these compounds lack sufficient specificity and are therefore toxic to the cell. The non-nucleoside antagonists of HIV-1 RT, on the other hand, are specific, noncompetitive inhibitors (with respect to dNTP) that do not have the toxicity problem that is associated with nucleoside inhibitors. These compounds include derivatives of thiobenzimidazolone, nevirapine, pyridinone, and bisarylpiperazine (14, 15, 16, 17, 18). Competition experiments have led to the suggestion that all of these inhibitors are likely to bind at the same or overlapping sites on HIV-1 RT (15,16,19,20).

The pyridinone inhibitors (16) of HIV-1 RT have been shown to inhibit the spread of HIV-1 infection in human T-lymphoid cell culture by at least 95% at concentrations in the range of 12 to 200 nM (17). However, the *in vitro* potency of these compounds depends on the sequence of the primer-template used in the polymerization assay. For example, the inhibition constant obtained for L-697,661 (Figure 1), a pyridinone derivative being examined in Phase II clinical trials, ranges from a high of 830 nM to a low of 19 nM depending on whether oligo(dT)·poly(rA) or oligo(dG)·poly(rC) is used as the primer-template for polymerization (17).

To better understand the effect of primer-template structure on the inhibitory potency of non-nucleoside inhibitors, we have prepared a number of heteromeric RNA templates whose sequences are based on that of the HIV-1 genome. The use of these RNA templates should more accurately model the conditions encountered by RT *in vivo*. We have found that under the condition of single-base incorporation the inhibitory potency of the pyridinone derivatives is dependent on the primary structure of the template but not on the identity of the incoming nucleotide (21). In this study, we have examined the effect of template secondary structure on the inhibitory potency of a non-nucleoside pyridinone inhibitor against *in vitro* reverse transcription catalyzed by HIV-1 RT.

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MATERIALS AND METHODS

Enzymes

Avian myeloblastosis virus (AMV) reverse transcriptase (2.5 units/ml) was purchased from Bethesda Research Laboratories and polynucleotide kinase (30 units/μl) was obtained from United States Biochemicals. HIV-1 reverse transcriptase was purified as previously described (21).

Reagents

Ultrapure deoxy- and dideoxy-nucleoside triphosphates (100 mM solutions) and oligo(dT)·poly(rA) were obtained from Pharmacia, Inc. The γ-^{32}P-labeled ATP was purchased from New England Nuclear. Formamide was obtained from Boehringer Mannheim. The pyridinone compound L-697,661 (16) was kindly supplied by J.Wai (Merck Research Laboratories, West Point). Rabbit Globin mRNA (50 μg/ml) was purchased from Gibco BRL.

Preparation of DNA and RNA oligomers

DNA deoxyoligonucleotides were prepared using either an Applied Biosystems Model 380B or 394 synthesizer. All DNA primers were purified by denaturing 20% polyacrylamide gel electrophoresis (PAGE). The DNA was isolated from the gel by crushing the gel piece followed with the addition of 1 ml of 0.3 M sodium acetate and mixing at 4°C overnight. Gel residue was removed by centrifugation in a microcentrifuge at 14 000 rpm for 15 min. The oligonucleotide was then precipitated with the addition of 2 volumes of absolute ethanol and incubation at −70°C for 12 h. Stock oligonucleotide solutions were prepared with the addition of 10 mM Tris−HCl, pH 7.8, to a final concentration of 20 μM. RNA oligonucleotides were synthesized as previously described (21). Concentrations of DNA and RNA oligonucleotides were determined by absorbance at 260 nm using extinction coefficients based on the nucleotide composition (22). Annealing of complementary primer and template was carried out by mixing ca. 110 nmol of each oligonucleotide along with a 0.2 nmol of 5′-[^{32}P]-end-labeled primer in 50 μl of 10 mM Tris−HCl, pH 7.8, for 5 min at 25°C. Table 1 lists the DNA and RNA sequences employed in this study.

Analysis of polymerization products using β-globin mRNA as template

Annealing of a 5′-[^{32}P]-end-labeled primer p1 (2 nmol) or p2 (see Table 1) with 2 μg of rabbit globin mRNA was carried out in a 50 μl reaction containing 10 mM Tris−HCl, pH 7.8. The solution was heated to 50°C for 3 min before being cooled to 25°C over 5 min. Primer-extension reactions catalyzed by RT were performed at 25°C in the presence of 50 mM Tris−HCl, pH 7.8, 70 mM KCl, 1 mM DTT, 3 mM $MgCl_2$, 0.2% (w/v) polyethylene glycol 8000, 150 μM deoxynucleoside triphosphates (dNTP), 5 μg/mL globin mRNA, 500 nM oligonucleotide p1 or p2, 20 nM HIV-1 RT, and 0, 33 or 333 nM L-697,661. Aliquots of the reaction mixture were quenched by the introduction of polyacrylamide gel electrophoresis (PAGE) loading buffer [90% formamide, 45 mM Tris−borate, pH 8.0, 20 mM EDTA, 0.1% (w/v) bromophenol blue and 0.1% (w/v) xylene cyanol FF]. Reaction products were separated with denaturing 8−20% PAGE and analyzed with standard autoradiography techniques. The build up of oligonucleotides of intermediate sizes at β-globin mRNA sequences corresponding to template secondary structures was quantified with the use of scanning laser densitometry (Pharmacia LKB Ultroscan XL equipped with Gelscan XL software).

Analysis of polymerization products using synthetic RNA templates and 5′-[^{32}P]-end-labeled primer p3

Reactions were performed using 500 nM annealed primer-template, 5 nM HIV-1 RT, 500 nM of dNTPs, 50 mM Tris−HCl, pH 7.8, 30 mM KCl, 1 mM dithiothreitol, 10 mM $MgCl_2$, and 0.2% polyethylene glycol 8000. Reactions were initiated with the addition of dNTPs. Aliquots of 6 μl were removed at intervals of 0.5, 5, 15, and 60 min and mixed immediately with PAGE loading buffer. Product analysis was accomplished by separation of the substrate 11-mer primer from the reaction products with denaturing 20% PAGE.

IC_{50} determinations

Reactions used for the determination of IC_{50} values contained 500 nM annealed primer-template, 1 to 5 nM HIV-1 RT, 0 to 823 nM L-697,661, 5 μM dNTP, 50 mM Tris−HCl, pH 7.8, 30 mM KCl, 1 mM dithiothreitol, 10 mM $MgCl_2$, and 0.2% (w/v) polyethylene glycol 8000. In some reactions, a 2-to-1 molar ratio of DNA-to-RNA oligonucleotides (Table 1) was used. Catalysis was initiated with the addition of dNTPs (5 μM each) after a 5 min preincubation of enzyme in the reaction mixture. All assays were carried out at 25°C unless otherwise stated.

Trapping experiments using β-globin mRNA

Trapping experiments were performed using modifications of a method previously described (23). Primer-extension reactions were carried out using β-globin mRNA and the 5′-[^{32}P]-end-labeled primer p2 in the buffer system described above. The trap (i.e., primer-template) added to the reactions consisted of 333 or 666 μg/ml oligo(dT)·poly(rA). In all of the reactions HIV-1 RT was first preincubated with β-globin mRNA in the presence or the absence of L-697,661 (33 or 166 nM) for 5 min at 25°C. Reactions were initiated with the addition of dNTPs alone or dNTPs plus unlabeled trap. A control reaction was carried out in which the primer-template trap was included in the preincubation mixture.

Molecular modelling

All of the primer-templates shown in Figure 6 were constructed using Macromodel (24). The protein data bank file 1D16 (25) was used as a guide to construct the loop region of primer-templates A, D and E. The primer-template structures were then energy minimized using Batchmin (24). The Klenow fragment (1DPI) (26) was retrieved from the protein data bank. The primer-templates were docked into the Klenow fragment by placing the 3′ end of the primer close to the catalytic site (Asp710, Asp882, and Glu883) and then removing any steric contacts between the primer-template and the rest of the Klenow fragment. The docking arrangement of the primer-template is similar to that of Besse *et al.* (27).

Data analysis

Nonlinear regression calculations were performed with the commercial computer program Kaleidagraph® formulated with the algorithm of Marquardt (28). For determinations of IC_{50} values (the concentration of inhibitor that confers 50% inhibition under given conditions), saturation activity data were fitted iteratively to Equation 1,

$$Y = \frac{(1-\alpha)\cdot[I]_t}{[I]_t + IC_{50}} \quad (1)$$

where Y is the fraction of activity inhibited, α is the residual activity at infinite concentration of inhibitor, and $[I]_t$ is the total concentration of inhibitor.

RESULTS

β-globin mRNA and pause sites

To observe the effect of secondary structure of the RNA template on the action of HIV-1 RT, reverse transcription catalyzed by the enzyme was characterized with the use of β-globin mRNA (29). The β-globin mRNA was primed with a 5'-[^{32}P]-end-labeled 17-mer DNA oligonucleotide at its polyA region from base 519 to base 535 (Figure 2). As seen in the DNA sequencing gel shown in Figure 3, the DNA primer was elongated with the incorporation of deoxynucleotides up to base position 488 of the template. The enzyme paused at this site (resulting in an accumulation of primer-extension product) before it carried out further catalysis of DNA polymerization (resulting in the eventual dissipation of this intermediate). The relative amount of heteropolymeric intermediate formed in the presence and absence of inhibitor at the pause site was quantified after autoradiography with scanning laser densitometry from another gel (containing more time points). The results are summarized in Figure 4. In the presence of L-697,661 the amount of intermediate built up at the pause site was not only greater but also decayed much more slowly. Control reactions using AMV reverse transcriptase also revealed a moderate pause in DNA synthesis at position 488; however, the intensity or duration of this pause was not influenced by the presence of the inhibitor (data not shown).

Synthetic heteromeric templates and pause sites

Examination of the β-globin mRNA sequence in the vicinity of nucleotide position 468 to 488 revealed two sequences of consecutive C and G bases that could be involved in the formation of a stem-loop hairpin structure (Figure 2). For this reason, the HIV-1 genome was searched for the sequence motif of 5'-GGGG-$(N)_{(4-10)}$-CCCC-3'. A match was found in the *pol* gene region from nucleotide base 1734 to 1770 (30). Using this sequence, a synthetic 37-mer RNA, template tR4, was prepared (see Table 1). Three derivatives of the tR4 RNA template were also synthesized and used as controls. With templates tR5 and tR7, the poly(C) or poly(G) sequence of template tR4 was disrupted so that a stem-loop structure could not be formed. With template tR6, the poly(G) and poly(C) bases of tR4 involved in the formation of the stem portion of the hairpin were interchanged.

Using the wild-type tR4 template sequence, we found that extension of an 11-mer DNA primer (p3, Table 1), annealed to the 3'-end of the template, was characterized by a pause at the nucleotide base 3' to the repeated C sequence at positions 20–22 of the template. Similarly, extension of the same primer on template tR6, which also had the potential to form a hairpin structure, was characterized by a pause at the positions 19–21, 3' to the repeated G sequence. These results are shown in Figure 5. To examine whether the pause observed in RT polymerization was indeed due to a duplex stem structure and not simply due to the presence of a single-stranded repeated C or repeated G sequence, we annealed the primer c5 to template tR7 to generate a heteroduplex on the 5'-end of this template. Using this combination, extension of the p3 primer from the 3'-end of tR7 yielded a pause at positions 19–21 of the template (data not shown). In contrast, no pause was observed for the extensions of primer p3 on tR7 or tR5 when these templates did not contain secondary structures (Figure 5). In the presence of L-697,661, the amount of intermediates accumulated at the pause sites of the synthetic RNA templates was again greatly enhanced, as observed previously using β-globin mRNA as template. The DNA sequencing gel data are not shown, but quantitative results, in terms of IC_{50} values, are summarized in Figure 6.

Trapping experiments

To examine whether L-697,661 affected the ease of dissociation of RT at pause sites, HIV-1 RT was preincubated with β-globin RNA annealed to a labeled 17-mer primer (p2) in the presence or absence of the inhibitor. Primer-extension was initiated by the simultaneous addition of dNTPs and a large excess of unlabeled oligo(dT)·poly(rA) as a trap. Comparison of the products from polymerization reactions B and E, depicted in Figure 3, revealed that, in the absence of inhibitor, but in the presence of trap, there was a significant increase in the intensity of the band at position 488. Reverse transcriptase that dissociated from the primer-template in the presence of trap would have been unable to extend the labeled primer further because it would bind to the excess unlabeled oligo(dT)·poly(rA). Although the intensity of labeled full-length products increased with time, the intermediate band at position 488 remained relatively constant in the presence of the trap as shown in Figure 3E, indicating that there was a significant amount of enzyme that dissociated at the pause site as well as some that translocated past template position 488 without dissociation.

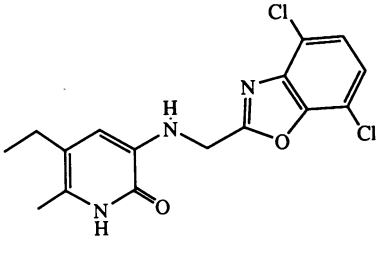

Figure 1. Structure of the HIV-1 specific non-nucleoside inhibitor L-697,661, 3-(4,7-dichlorobenzoxazol-2-ylmethylamino)-5-ethyl-6-methylpyridin-2(1H)-one.

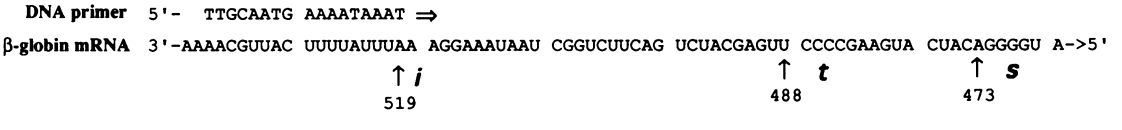

Figure 2. Sequence of the first seventy bases of the 3'-region of β-globin mRNA primed with p2, a 17-mer primer. The symbols *i*, *t*, and *s* represent, respectively, the start of primer-extension, the pause site position, and the region of 4 Gs that might be involved in the formation of a hairpin by base pairing to the Cs in the *t* region.

Table 1. Sequence of synthetic RNA and DNA oligonucleotides used as primer-template combinations in the HIV-1 RT catalyzed reverse transcription reactions

A. Four 37-mer synthetic RNA with sequences based on that of HIV-1 genome[a]

	1734 ↓	1770 ↓
tR4 Wild-type[b]	5'-GGGGAAGAGA UAACCCCCUC UCAAAAACAG GAGCAGA -3'	
tR5 oligo-C abolished[c]	5'-GGGGAAGAGA UAACUAGCUC UCAAAAACAG GAGCAGA-3'	
tR6 swap Cs & Gs[b,c]	5'-CCCCAAGAGA UAAGGGGGUC UCAAAAACAG GAGCAGA-3'	
tR7' oligo-G abolished[c]	5'-CUAGAAGAGA UAACCCCCUC UCAAAAACAG GAGCAGA-3'	

B. DNA primers used in combination with the RNA templates

p1	5'-TTT TGC AAT G -3'
p2	5'-TTG CAA TGA AAA TAA AT -3'
p3	5'-TCT GCT CCT GT -3'
p4	5'-TCT GCT CCT GTT TTT-3'

C. Complimentary DNA oligonucleotide used to block RT synthesis on tR7

c5	5'-GGG GGT TAT CTC TTC TAG-3'

[a] HIV-1 genome sequence numbering according to ref. (30).
[b] Bases underlined are predicted (36) to be involved in the base paired stem of a hairpin loop (see Figure 6).
[c] Bases in bold have been changed from those in the HIV-1 genome sequence.

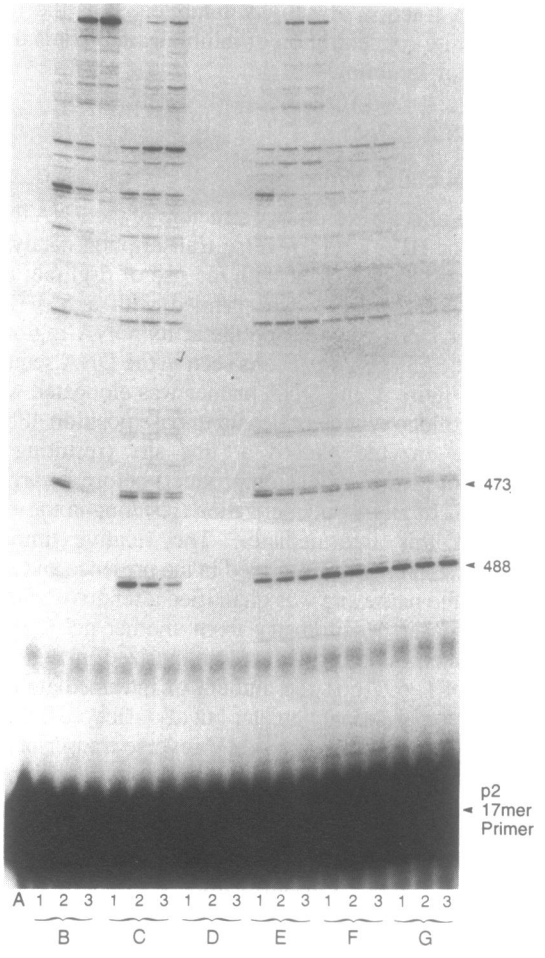

Figure 3. Polyacrylamide gel electrophoretic analysis of intermediates and products of reverse transcription in reactions catalyzed by HIV-1 RT. The template used was β-globin mRNA and the reactions were conducted as described in Methods in the presence and absence of L-697,661 and the trap primer-template oligo(dT)·poly(rA). Aliquots of the reaction mixture were removed after 10, 30, and 60 min (1−3) and added to PAGE loading buffer before electrophoretic separation on a 10 % polyacrylamide gel. Intermediates corresponding to the pause products at positions 488 and 473 (see Figure 2) are indicated with an arrow. Individual primer-extension reactions contained: *A*. no RT; *B*. RT; *C*. RT, 33 nM L-697,661; *D*. RT, 333 μg/ml oligo(dT)·poly(rA) added before RT (Trap control); *E*. RT added with 333 μg/ml oligo(dT)·poly(rA); *F*. RT added with 333 μg/ml oligo(dT)·poly(rA), 33 nM L-697,661; *G*. RT added with 333 μg/ml oligo(dT)·poly(rA), 166 nM L-697,661. The pause site location on the template RNA was determined by dideoxy sequencing using HIV-1 RT.

In the presence of L-697,661, but in the absence of the oligo(dT)·poly(rA) trap, the intensity of the band at position 488 significantly increased with respect to the intensity of the corresponding band obtained in the uninhibited reaction. As shown in Figure 3C, in the presence of inhibitor the pause band at position 488 decreased over time with a concomitant increase in full-length product. The results obtained upon addition of the oligo(dT)·poly(rA) trap to the reaction was similar to that observed for the uninhibited reaction as shown in Figure 3F and G. There was a time dependent increase in full-length product and the intensity of the band at position 488 remained relatively constant. In contrast, the trap was shown to be effective in a control reaction in which RT was preincubated in the presence of excess oligo(dT)·poly(rA) (see Figure 3D). There was no observable extension of the primer with time.

Inhibitory potency of L-697,661

Quantitative analyses of the rate of extension of the 11-mer primer to various positions of the above mentioned synthetic heteromeric templates allowed an assessment of the inhibitory potency of L-697,661 as affected by the secondary structures of the template. Two sets of IC_{50} values were measured for the inhibitor.

Inhibition by L-697,661 of a reaction incorporating four dTMP groups onto the 3'-end of p3 was determined using templates tR4, tR6, tR7, and template tR7 annealed with c5 (to provide a duplex structure without a hairpin loop downstream from the nascent chain). The inhibitory potency of L-697,661 was higher for templates tR4 and tR6 than for tR7. The potency of L-697,661 obtained using tR7 (with or without the complementary oligonucleotide c5) was approximately half those of the templates with hairpin structures. These results are summarized in Figure 6.

The potency of L-697,661 was also determined for RT-catalyzed primer-extension to nucleotide position 28 of templates tR4, tR7, and template tR7 annealed with c5 by providing in the reaction mixture dTTP, dGTP, and dATP. To extend p3 to nucleotide position 28, the hairpin structure of template tR4 and the duplex structure of template tR7 annealed with c5 needed to be disrupted, so the inhibitory potency of L-697,661 was expected to be higher. IC_{50} values of < 10 nM and 14 nM were found for inhibition by L-697,661 of primer-extension to position 28 of template tR4 and template tR7 annealed with c5 (Figure 6A and C), respectively. In contrast, an IC_{50} of 67 nM was found for tR7 (without c5) (Figure 6B). There is no difference in the sequence of templates tR4 and tR7 up to nucleotide position 28. The dNTP combination used for the IC_{50} determination with tR7 and tR4 could not be applied to tR6 due to the reversal of the cytosine and guanine bases which formed the stem of the

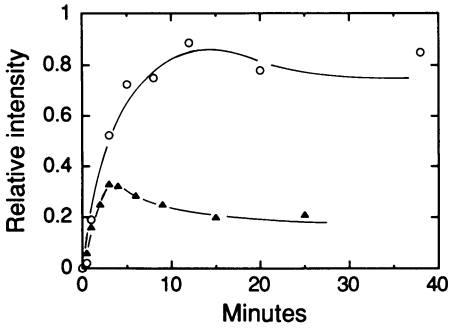

Figure 4. Time course of pause-site intermediates generated in reactions catalyzed by HIV-1 RT at nucleotide base position 488 of β-globin mRNA in the presence and absence of L-697,661. Primer-extension reactions were performed as described in Methods using primer p1. Aliquots were removed after 30s to 65 min and added to PAGE loading buffer. Products were separated on a 20% polyacrylamide gel, and the bands corresponding to the pause at positions 488 and 473 were quantified by scanning laser densitometry and plotted against time for reaction A (open symbols, no inhibitor added) and reaction B (closed symbols, 330 nM L-697,661 present).

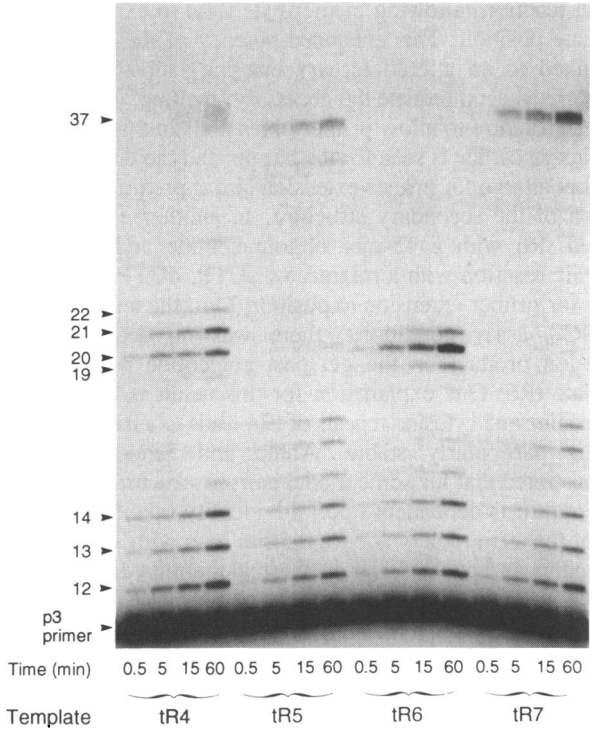

Figure 5. Polyacrylamide gel electrophoretic analysis of intermediates and products of reverse transcription in reactions catalyzed by HIV-1 RT. The templates used were heteromeric (Table 1) and the reactions were conducted as described in Methods. Bands corresponding to the 11-mer primer (p3) and elongation products up to the full-length 37-mer product are identified at the left side of the gel.

hairpin structure. We determined the potency of L-697,661 against synthesis on template tR6 using a mixture of dCTP, dGTP and dATP along with the 15-mer oligonucleotide primer p4. With this substrate combination, RT had the potential to catalyze extension of the primer to position 24 of the template (Figure 6D). However, no primer-extension was observed past position

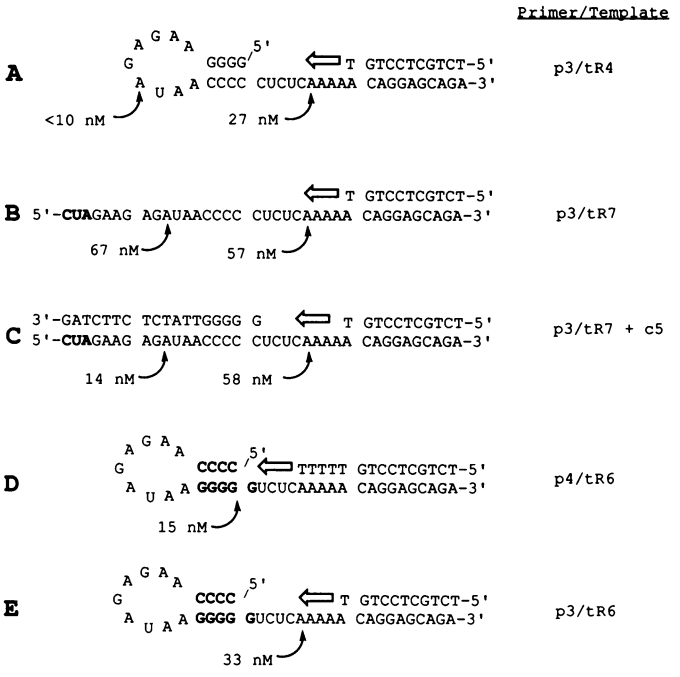

Figure 6. A summary of the inhibitory potency of L-697,661 (IC_{50}) for several primer-templates (with and without secondary structures) and different extents of polymerization in reverse transcriptions catalyzed by HIV-1 RT. The solid arrows indicate the termination point of each primer-extension as limited by the combination of dNTPs supplied to the reaction. The different primer-template and dNTP combinations, together with the respective IC_{50} values, are: *A*. p3-tR4, dTTP (IC_{50} = 27 nM) or dATP+dGTP+dTTP (IC_{50} < 10 nM. Experiments to determine the IC_{50} for this primer-template combination required 1 nM RT in order to obtain significant quantities of product for accurate quantification. Since the concentration of L-697,661 was not in large excess of the concentration of RT, we conservatively conclude that the IC_{50} is < 10 nM.). *B*. p3-tR7, dTTP (IC_{50} = 57 nM) or dATP+dGTP+dTTP (IC_{50} = 67 nM). *C*. p3-tR7 + c5, dTTP (IC_{50} = 58 nM) or dATP+dGTP+dTTP (IC_{50} = 14 nM). *D*. p4-tR6, dATP+ dCTP+dGTP (IC_{50} = 15 nM. There was no observable polymerization through the pause site using this primer-template. The actual number of bases incorporated was 4 to 5.). *E*. p3-tR6, dTTP (IC_{50} = 33 nM). We have depicted only one mode of base pairing in the stem of the hairpin templates. It is probable that there is a population with base pairing to bases 22–24 (three base pairs) or to bases 20–23. This may account for the multiple pause site bands observed in Figure 5.

21. An IC_{50} for the inhibition by L-697,661 of synthesis to position 21 was determined to be 15 nM.

DISCUSSION

DNA polymerization experiments conducted using β-globin mRNA as template revealed that a specific pattern of primer-extension products can be generated presumably due to differential dissociation of HIV-1 RT from the template during processive synthesis. The results, given in Figure 3B and C, show that using our assay conditions there is a build up of an intermediate-length product associated with the presence of a group of four consecutive cytosines on the RNA template giving rise to an apparent 'pause' during reverse transcription. Furthermore, this pause in the primer-extension process appears to limit the amount of full-length product. Figure 4 shows that in the presence of the non-nucleoside inhibitor a much larger amount of intermediate is accumulated at the pause site and that

the rate at which this intermediate dissipates is greatly decreased. As expected, the effect of L-697,661 is HIV-1 RT specific since this class of non-nucleoside compounds inhibits only HIV RT; the pause in DNA synthesis catalyzed by AMV RT is not affected by L-697,661.

Since the pause appears to be isolated to a region of consecutive cytosine bases, it seems reasonable to speculate that RT-catalyzed polymerization is slowed by this specific primary structure of the template (21, 31). It is also possible that another region of the globin RNA folds back onto the four cytosines resulting in the formation of a stem-loop secondary structure. This latter hypothesis is particularly attractive since there is a stretch of five consecutive guanine nucleosides on the template only ten bases downstream (in the direction of primer-extension) from the cytosine nucleosides which could participate in the formation of a hairpin structure. (There are several pause sites on β-globin RNA. Only one of which is studied here to identify sequences that introduce polymerization pauses for the purpose of modelling pause sites with use of synthetic RNA oligonucleotides.)

To test whether template secondary structure is responsible for transcriptional pauses, a synthetic RNA template, tR4, was employed together with its three derivatives tR5, tR6, and tR7 (Table 1). Template tR4 contains the 5′-GGGG-$(N)_{(4-10)}$-CCCC-3′ motif within a sequence corresponding to nucleotide bases 1734−1770 in the *pol* stretch of the *Eli* HIV-1 genome isolate (30). PAGE analyses of the primer-extension products in RT-catalyzed reactions containing these templates clearly reveal that the templates capable of forming a hairpin secondary structure (tR4 and tR6) downstream from the site of polymerization give rise to reverse transcriptional pauses. Also, tR7 annealed with oligodeoxynucleotide c5 to its 5′ end, a template that contains a duplex structure downstream from the site of polymerization, also yields pauses. The results are dark bands on the sequencing gel corresponding to accumulation of primer intermediates extended to bases 19−21 of the template (Figure 5). On the other hand, the presence of 4 to 5 consecutive cytosine bases alone (tR7) does not result in a transcriptional pause. These results indicate that the stem portion and/or the loop portion of a hairpin secondary structure of the RNA template can lead to strong hindrance of reverse transcription in the HIV-1 RT catalyzed reaction.

To examine the effect of a hairpin structure on the inhibition of pyridinone derivatives, IC_{50} values have been determined in reactions for L-697,661 against RT-catalyzed DNA polymerization to either a site upstream from or into the hairpin domain of the RNA template (Figures 5 and 6). L-697,661 inhibition of primer-extension to a template base several nucleotides away from a hairpin stem-loop structure (tR4 and tR6, Figure 6A and E) is diminished by a factor of two if the hairpin is absent (tR7, Figure 6B). Interestingly, the presence of a duplex template structure in the absence of a loop structure does not affect the inhibitory potency of L-697,661 on RT-catalyzed base incorporation away from the secondary template structure as seen in the case of template tR7 annealed with c5 to its 5′-end (Figure 6C). One explanation for this is that the loop moiety but not the stem moiety of the hairpins of templates tR4 and tR6 interacts with HIV-1 RT in a manner that affects the binding of L-697,661 and, thereby, its inhibitory potency. To evaluate if this is a plausible explanation, we have modeled a primer-template into the active site of the Klenow fragment of *E.coli* DNA polymerase I. (The X-ray coordinates of HIV-1 RT are not yet publicly disclosed ao we chose the most closely related model system available to help us formulate an explanation for our results.) This protein has been reported (32) to possess a structure similar to HIV-1 RT. With the 3′-end of the primer placed in the vicinity of the active-site Asp^{710}, Asp^{882}, and Glu^{883} residues, the hairpin of template tR4 cannot thread through the cavity between the 'fingers' and 'thumb' domains of the enzyme without protein conformational changes; the loop moiety of the hairpin is in direct van der Waals contact with the 'fingers' domain of this enzyme, thus hindering translocation. In contrast, our modeling indicates that a duplex structure such as c5 annealed to template tR7 can thread through the active site of Klenow for processive synthesis. Based on this model we speculate that steric interactions, caused by the loop moiety of a hairpin structure on the RNA template and the 'fingers' domain of HIV-1 RT, may be responsible for the increase in the inhibitory potency of L-697,661 on RT-catalyzed base incorporation adjacent to the template secondary structure, if such interactions affect the binding of the pyridinone compound.

For primer-extension catalyzed by HIV-1 RT through the pause and into the hairpin moiety of the RNA template, the inhibitory potency of L-697,661 is significantly increased (Figure 6). The observed IC_{50} of ~10 nM for templates tR4 and tR7 (annealed with c5) may be compared with an IC_{50} of 67 nM for template tR7 in reactions allowing primer-extension to exactly the same template position. This enhanced potency of the inhibitor may be linked to an altered ternary enzyme−substrate−inhibitor complex to accommodate the necessary 'melting' of the template duplex structure to allow primer-extension. In this case, similar inhibitory potency is seen for the hairpin and the duplex structure (without a loop) for primer-extension into approximately the mid-section of the secondary structure. In another experiment we primed tR6 with a 15-mer oligonucleotide and initiated the enzymic reaction with a mixture of dCTP, dGTP and dATP to allow for primer-extension to position 24 of the template. Under our IC_{50} assay conditions, there was no detectable DNA extension product on the gel past nucleotide position 21 on template tR6. One explanation for this result is that reversing the guanine and cytosine repeats of tR4 leads to a hairpin structure that is particularly stable. Antao and Tinoco (33) have demonstrated that for some model hairpin structures the melting temperature is dependent upon the identity of the closing base pair of the stem moiety (i.e., a hairpin loop with a closing G·C base pair can have a different thermal stability from that with a C·G base pair) as well as the identity of the base pair that is upstream in the sequence.

Recently, Klarmann *et al.* (31) identified and characterized *in vitro* generated RT pause sites using HIV-1 genome derived sequences. In their work, the authors correlated the production of pause sites with runs of rGs, rCs and the presence of RNA secondary structures such as hairpins. This correlates well with our results for the location of the pause using β-globin mRNA. In addition, Klarmann *et al.* proposed two mechanisms by which RT might pause at a particular template position based on oligo(dT)·poly(rA) trapping experiments. The first mechanism requires RT to dissociate from the template. The second is that the enzyme remains bound to the template but polymerization continues through the pause at a rate that is much slower than the normal synthesis rate. Both of these are mechanistic extremes and the actual reason might be due to some combination of the two.

We have considered these two possibilities. If the latter mechanism is responsible for the pause observed in our

experiments, then one would expect the pause band to decrease in intensity over time with a concomitant increase in longer-length products (in the absence or presence of trap). However, we observed an increase in intensity of the longer-length products but the pause band retained the same intensity over the time course of the experiment. One possibility which is consistent with our results considers that only a certain population of the RNA contains the structure that is responsible for the pause. As the enzyme encounters the structure, the rate of translocation through the pause is very slow making dissociation of the RT·primer-template complex more kinetically significant. The presence of the inhibitor apparently accentuates this effect.

Koop *et al.* (34) have also studied the effect of the non-nucleoside inhibitor dipyridodiazepinone, BI-RG-587, on processivity of HIV-1 RT using a heteromeric RNA template whose sequence is 60 bases in length and corresponds to the HIV-1 $tRNA^{Lys}$ primer binding region. In the presence of BI-RG-587, these investigators found that the amount of DNA products of all lengths was diminished to an equal extent in reverse transcription catalyzed by the enzyme. We are unable to identify any regions of stable secondary structure within this 60-base sequence of the HIV-1 genome and, therefore, do not expect a pause in the action of RT on this template during catalysis. However, using BI-RG-587 in conjunction with the β-globin mRNA, we have been able to demonstrate that this non-nucleoside inhibitor of HIV-1 RT can also induce an enhanced pause, at position 488 of the template, in a manner similar to that observed for L-697,661 (data not shown).

Several previous reports have focused on the significance of HIV-1 RT·primer-template interactions. As pointed out by Kenyon *et al.* (35), the use of homopolymer oligo(dT)·poly(rA) as primer-template in the assay of inhibitor potency may not be a sufficiently realistic system for RT-polymerization *in vitro*. We have shown that the potency of non-nucleoside inhibitors is dependent on the primary structure of the RNA template (21). Recent X-ray crystallography data of HIV-1 RT also implicates the potential importance of enzyme/template interactions. Kohlstaedt *et al.* (32) have shown that the amino acids involved in RT resistance to AZT cluster in two regions of the protein that are thought to interact with template RNA. The authors suggest that the discrimination between the modified nucleotides such as AZT and dideoxyinosine might be governed by the interaction of enzyme and template. The results presented here also implicate that interactions of HIV-1 RT with the secondary structure of the primer-template may play a significant role in its inhibition by non-nucleoside inhibitors.

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